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## **Identification of a naturally processed NY-ESO-1 peptide recognized by CD8+ T cells in the context of HLA-B51**

Jäger, Elke ; Karbach, Julia ; Gnjjatic, Sacha ; Jäger, Dirk ; Maeurer, Markus ; Atmaca, Akin ; Arand, Michael ; Skipper, Jonathan ; Stockert, Elisabeth ; Chen, Yao-Tseng ; Old, Lloyd J ; Knuth, Alexander

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## Identification of a naturally processed NY-ESO-1 peptide recognized by CD8+ T cells in the context of HLA-B51

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**Keywords:** human, cytotoxic T lymphocytes, HLA-B51, NY-ESO-1, peptides

## Abstract

NY-ESO-1 is one of the most immunogenic cancer antigens known to date, inducing humoral and cellular immune responses in a high proportion of patients with advanced NY-ESO-1-expressing cancers. The assessment of spontaneous and vaccine-induced CD8+ T cell responses has been limited to a small number of known NY-ESO-1 epitopes presented by MHC class I alleles. Recently, a new method to monitor NY-ESO-1-specific CD8+ T cell responses was introduced that does not depend on the individual MHC class I status and on predefined peptide epitopes. Antigen-presenting cells transduced with recombinant adenoviral vectors encoding NY-ESO-1 were used to stimulate CD8+ selected NY-ESO-1-specific T cells. Effector cells were tested for recognition of autologous B cell targets transfected with NY-ESO-1 using a recombinant vaccinia virus construct. Using a modified approach we identified the NY-ESO-1 p94-102 peptide as being recognized by CD8+ T cells in the context of HLA-B51. NY-ESO-1 p94-102 specific CD8+ T cells recognized naturally processed NY-ESO-1 presented by HLA-B51+ monocyte-derived dendritic and tumor cells. Transfection of target cells with NY-ESO-1 combined with different HLA class I alleles confirmed that the NY-ESO-1 peptide was naturally processed and recognized by HLA-B51-restricted CD8+ T cell lines and clones. Therefore, NY-ESO-1 p94-102 is a new candidate peptide antigen for cancer immunotherapy and for the monitoring of spontaneous and vaccine-induced NY-ESO-1-specific T cell responses in HLA-B51+ patients with NY-ESO-1 expressing malignancies.

## Introduction

NY-ESO-1 was identified by serological analysis of a recombinant cDNA expression library (SEREX) from a squamous cell cancer of the esophagus (1). NY-ESO-1 belongs to the 'cancer/testis' family of antigens (2). NY-

ESO-1 expression is observed in different types of cancer, including hematological malignancies, and is restricted in normal tissues to germ cells (3, 4, 5, 6, 7, 8). NY-ESO-1 is one of the most immunogenic tumor antigens known to date, eliciting spontaneous humoral and cellular immune responses in approximately 50% of patients with advanced NY-ESO-1 expressing cancers (9, 10). Spontaneous immunity against NY-ESO-1 is dependent on the presence of NY-ESO-1+ disease (10). Increases or decreases in NY-ESO-1 serum antibody titers and frequencies of NY-ESO-1-specific CD8+ T cell responses strongly correlate with progression or regression of NY-ESO-1+ cancers (10, 11).

A number of sensitive methods have been developed to monitor spontaneous and vaccine-induced immune responses against NY-ESO-1 in cancer patients. NY-ESO-1 serum antibodies, as assessed by ELISA or Western blot techniques, were identified as reliable indicators for CD8+ T cell responses (9, 10). The latter are detected by complementary methods, such as HLA-A2/peptide tetramers, ELISPOT and cytotoxicity assays (11, 12).

Studies of spontaneous and vaccine-induced T cell reactivity against NY-ESO-1 are restricted to the limited number of NY-ESO-1 epitopes known to be recognized by CD8+ T cells. Three NY-ESO-1 peptides that are recognized in the context of HLA-A2 have been described (13). Additional NY-ESO-1 epitopes presented by HLA-A31 were defined that are encoded by the normal and the alternative open reading frame of NY-ESO-1 (14). To broaden the patient population that can be evaluated for NY-ESO-1-specific T cell immunity, a new analytical approach was introduced by Gnjjatic *et al.* which detects NY-ESO-1-specific CD8+ T cell reactivity independently of the predefined peptide epitopes presented by individual HLA class alleles. Antigen-presenting cells were transfected with adenovirus constructs encoding NY-ESO-1 to stimulate CD8+ selected effector cells. Autologous B cells were infected with vaccinia vectors encoding NY-ESO-1 and used as targets for specific CD8+ T cell recognition. The analysis of candidate NY-ESO-1 peptides presented by the respective HLA restriction elements has led to the identification of additional NY-ESO-1 epitopes presented by HLA-Cw3 and HLA-Cw6 (12). Applying this method, we identified a new NY-ESO-1 epitope that is recognized by CD8+ T cells from patients with detectable NY-ESO-1 serum antibody in the context of HLA-B51. CD8+ T cells recognized NY-ESO-1+, HLA-B51+ tumor cells presenting the naturally processed NY-ESO-1 p94-102 peptide. Therefore, the new NY-ESO-1 epitope is considered to be suitable for vaccination studies and the monitoring of NY-ESO-1-specific immune responses in HLA- B51+ patients with NY-ESO-1-expressing cancers.

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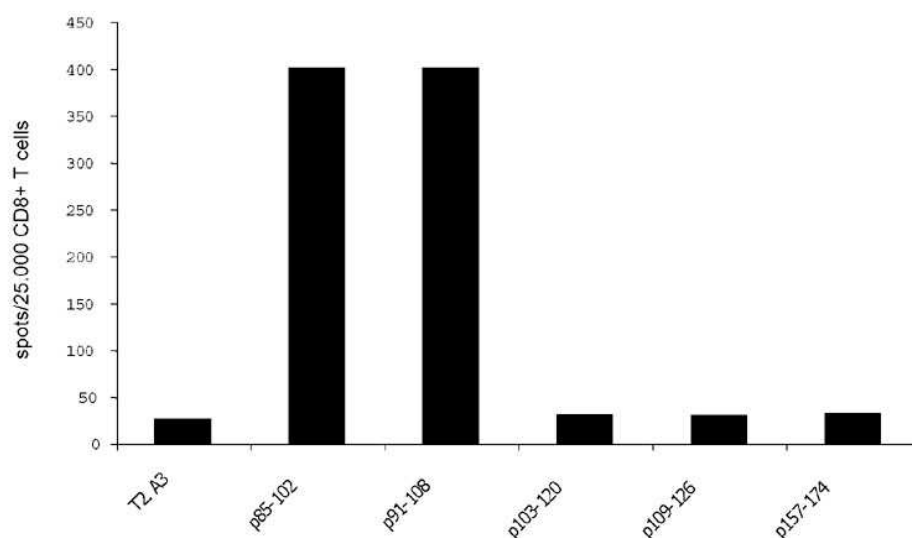
## Results

Based on our previous study showing a correlation between the humoral and cellular response against NY-ESO-1, five patients with NY-ESO-1-expressing tumors and who were seropositive for NY-ESO-1 were selected to determine the nature of their CD8+ T cell responses.

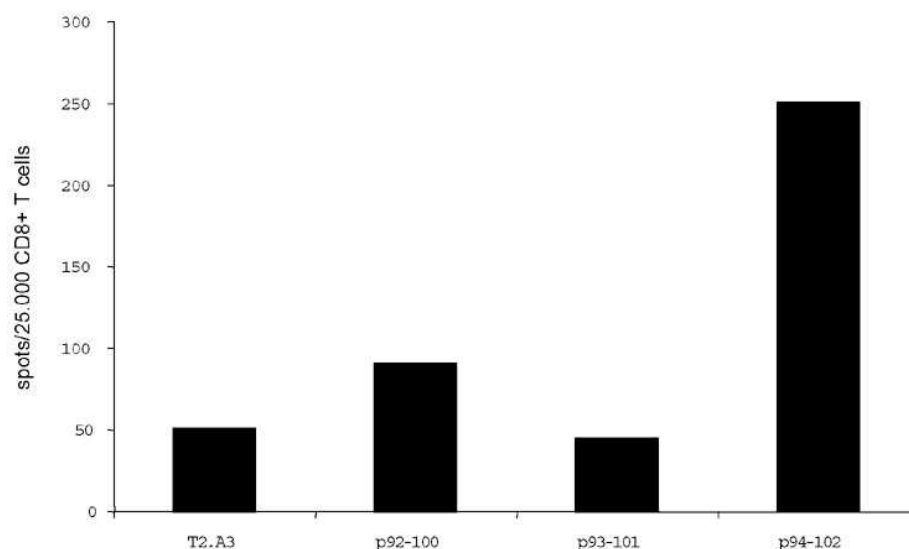
### **Sensitization with recombinant adenovirus encoding NY-ESO-1 induces specific CD8+ T cell responses against NY-ESO-1 peptides**

CD8+ selected effector T cells were sensitized with NY-ESO-1 recombinant adenovirus (Ad2/ESO)-infected, CD8-depleted PBLs of melanoma patient NW1539 and tested for the recognition of NY-ESO-1 epitopes on T2.A3 cells pulsed with long, overlapping 18-mer NY-ESO-1 peptides in ELISPOT assays. NY-ESO-1 peptides p85-102 and p91-108 were recognized (Figure 1). Since HLA-A3 and B51 were the only MHC class I alleles shared by the antigen-presenting T2.A3 cell line and the CD8+ effector T cells from patient NW1539, six candidate 9-mer peptides which can potentially bind to HLA-A3 or B51 and which are located within the 18-mer

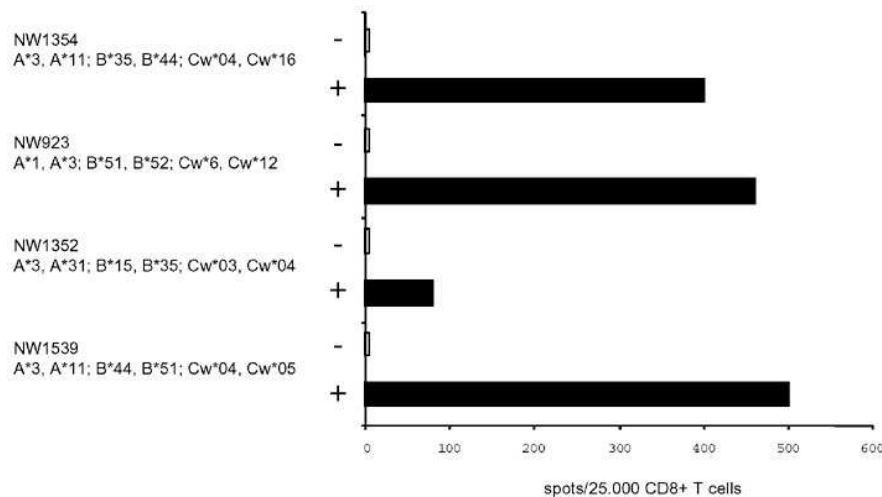
sequences recognized were synthesized and tested for recognition by Ad2/ESO-presensitized CD8+ NW1539 T cells. Figure 2 shows the results of an ELISPOT assay against T2.A3 target cells pulsed with NY-ESO-1 9-mer peptides recognized by CD8+ NW1539 T cells. A strong reactivity was demonstrated against NY-ESO-1 p94-102 (Figure 2). The specific reactivity against NY-ESO-1 p94-102 was confirmed by the analysis of peptide-presensitized CD8+ T cells derived from four patients who were seropositive for NY-ESO-1 and who share the HLA-A3 and/or the B51 allele (Figure 3).



**Figure 1. ELISPOT analysis of the recognition of overlapping NY-ESO-1 18-mer peptides by Ad2/ESO-presensitized CD8+ NW1539 T cells.** CD8+ T cells from patient NW1539 were presensitized for 6 days with T cell depleted, Ad2/ESO-infected, irradiated autologous PBLs. The presensitized cells ( $2.5 \times 10^4$  cells/well) were then incubated with irradiated T2.A3 cells ( $5 \times 10^4$  cells/well) pulsed with the indicated NY-ESO-1 peptides.



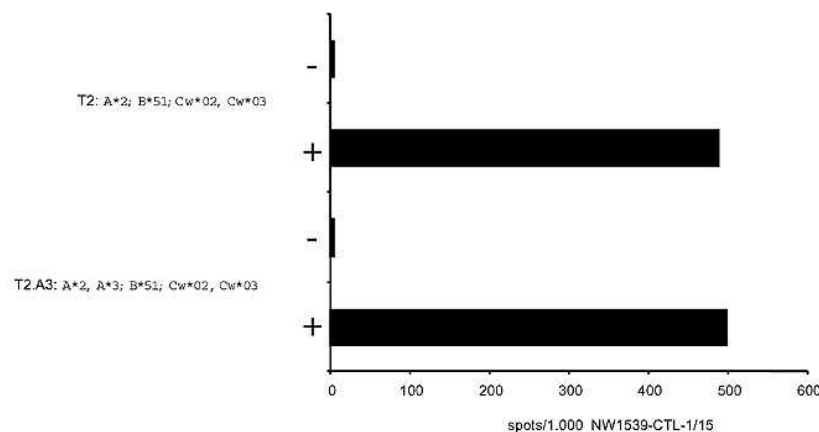
**Figure 2. ELISPOT analysis of the recognition of NY-ESO-1 p92-100, p93-101, and p94-102 peptides by Ad2/ESO-presensitized CD8+ NW1539 T cells.** CD8+ T cells from patient NW1539 were presensitized for 6 days with T cell depleted, Ad2/ESO-infected, irradiated autologous PBLs. The presensitized cells ( $2.5 \times 10^4$  cells/well) were then incubated with irradiated T2.A3 cells ( $5 \times 10^4$  cells/well) pulsed with NY-ESO-1 peptides p92-100, p93-101, or p94-102.



**Figure 3. ELISPOT analysis of the recognition of NY-ESO-1 p94-102 peptide by NY-ESO-1 p94-102 peptide-presentsitized CD8+ T cells from four NY-ESO-1 seropositive patients sharing the HLA-A3 and/or the B51 allele.** CD8+ T cells from patients NW1354, NW923, NW1352 and NW1539 were presensitized for 6 days with T cell depleted, p94-102 peptide-pulsed, irradiated PBLs. The presensitized cells ( $2.5 \times 10^4$  cells/well) were then incubated with irradiated, unpulsed (-) or NY-ESO-1 p94-102 peptide-pulsed (+) T2.A3 target cells ( $5 \times 10^4$  cells/well).

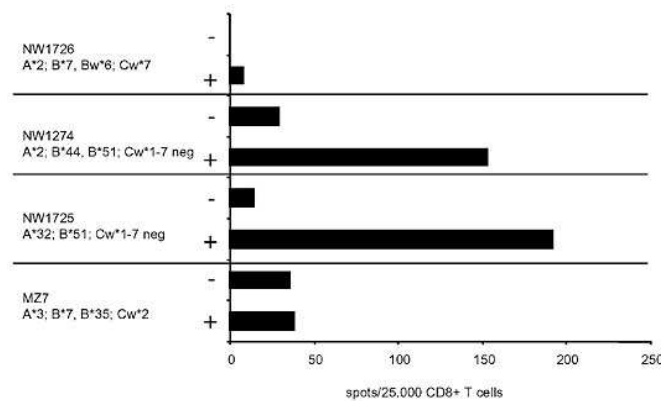
### Specific recognition of NY-ESO-1 p94-102 in the context of HLA-B51

The CD8+ T cell clone NW1539-CTL-1/15, obtained by repeated *in vitro* stimulations with the autologous tumor cell line NW-MEL-1539, was tested against NY-ESO-1 p94-102 peptide-pulsed T2 and T2.A3 target cells to discriminate between HLA-A3 and B51 as the restriction element. As shown in Figure 4, both peptide-pulsed T2 and T2.A3 cells were recognized with comparable efficacy, suggesting that NY-ESO-1 p94-102 recognition was not dependent on the presence of HLA-A3, leaving HLA-B51 as the potential restriction element for p94-102 presentation.



**Figure 4. ELISPOT analysis of the recognition of NY-ESO-1 p94-102 peptide by CD8+ T cell clone NW1539-CTL-1/15.** NW1539-CTL-1/15 T cells (1000 cells/well) were incubated with irradiated, unpulsed (-) or NY-ESO-1 p94-102 peptide-pulsed (+) T2 or T2.A3 cells ( $5 \times 10^4$  cells/well). Peptide-pulsed T2 and T2.A3 cells were recognized equally well, suggesting that HLA-B51 is the restriction element for T cell recognition.

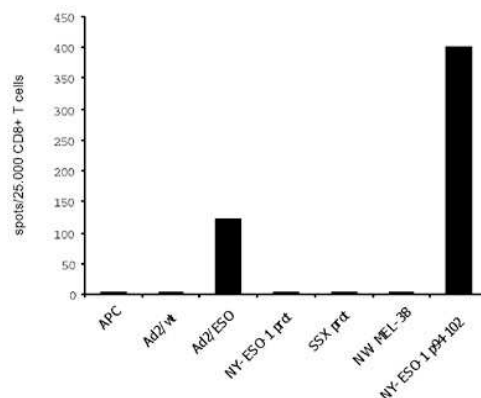
To confirm that NY-ESO-1 p94-102 was presented by HLA-B51, we tested peptide-pulsed PHA blasts derived from different donors as targets for recognition by CD8<sup>+</sup> T cells from melanoma patient NW1274 presensitized with NY-ESO-1 p94-102 peptide. Figure 5 shows a strong recognition of NY-ESO-1 p94-102 peptide-pulsed HLA-B51<sup>+</sup> target cells NW1725 and NW1274. No CD8<sup>+</sup> T cell reactivity was found against HLA-B51 negative target cells MZ7 and NW1726.



**Figure 5. ELISPOT analysis of the recognition of peptide-pulsed PHA blasts expressing different HLA alleles derived from cancer patients and healthy donors by NY-ESO-1 p94-102 peptide-presensitized NW1274 CD8<sup>+</sup> T cells.** PHA blasts were unpulsed (-) or pulsed with 10 µg/ml NY-ESO-1 p94-102 peptide (+). Recognition of HLA-B51<sup>+</sup> target cells NW1274 and NW1725 confirms the HLA-B51 restriction of T cell recognition.

### Recognition of naturally processed NY-ESO-1 by peptide-sensitized CD8<sup>+</sup> effector T cells

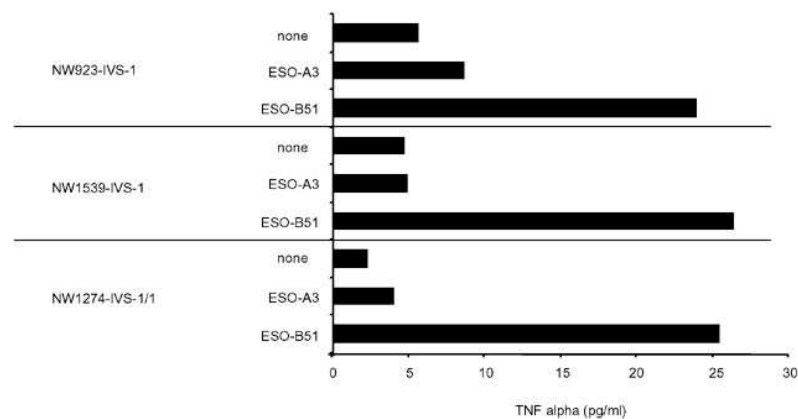
CD8<sup>+</sup> selected effector T cells of the HLA-B51<sup>+</sup> melanoma patient NW1539 were presensitized with NY-ESO-1 p94-102 and tested in ELISPOT assays on autologous, monocyte-derived APCs pulsed with the stimulating peptide or infected with Ad2/ESO. Figure 6 shows the specific recognition of NY-ESO-1 p94-102 and Ad2/ESO-transfected APCs, suggesting that NY-ESO-1 peptide p94-102 is naturally processed. APCs pulsed with the NY-ESO-1 recombinant protein, the SSX protein, or a lysate of the NY-ESO-1 expressing tumor cell line NW-MEL-38 were not recognized.



**Figure 6. Specific recognition of NY-ESO-1 p94-102 and Ad2/ESO-transfected APCs.** ELISPOT analysis of the recognition by NY-ESO-1 p94-102 peptide-presensitized NW1539 CD8<sup>+</sup> T cells of autologous, monocyte-derived APCs infected with wild-type or NY-ESO-1 adenovirus (30 pfu/cell) or pulsed with either 1 µg NY-ESO-1 and 1 µg SSX protein, a lysate of the NY-ESO-1<sup>+</sup> melanoma cell line NW-MEL-38 or NY-ESO-1 p94-102 peptide (10 µg/ml).

## Recognition of naturally processed NY-ESO-1 is restricted to HLA-B51

To ascertain the HLA class I restriction element for NY-ESO-1 p94-102 peptide-specific CD8<sup>+</sup> T cell responses against naturally processed NY-ESO-1, we tested COS-7 cells transfected with NY-ESO-1 and either HLA-A3 or B51. Figure 7 shows that the HLA-A3<sup>+</sup>, B51<sup>+</sup> effector T cell lines NW923 and NW1539 specifically recognize NY-ESO-1 in the context of HLA-B51, and not of HLA-A3. These results suggest that the NY-ESO-1 p94-102 epitope is naturally processed and presented only by HLA-B51.



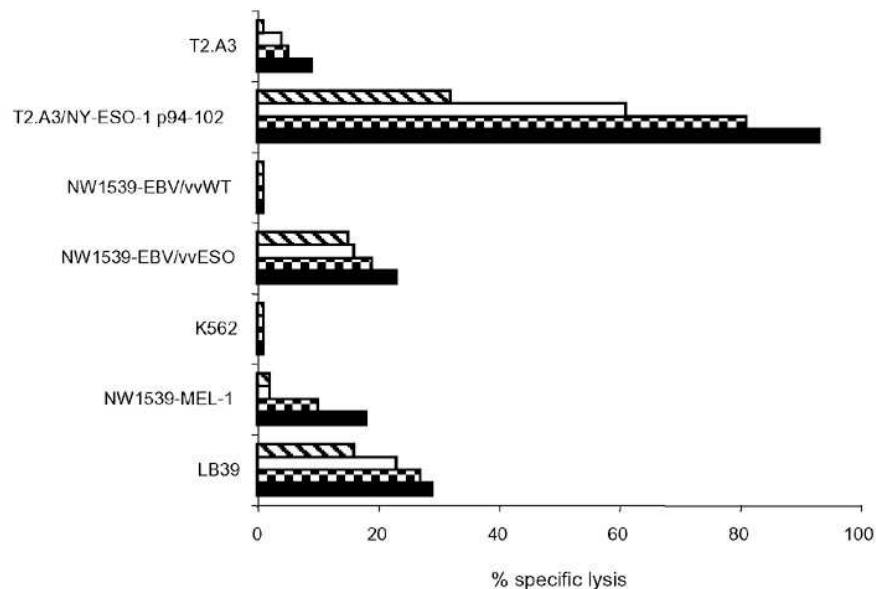
**Figure 7. TNF-alpha release assays following stimulation of the CD8<sup>+</sup> T cell lines NW923-IVS-1, NW1274-IVS-1 and NW1539-IVS-1 by COS-7 cell transfectants.** TNF-alpha release was detected after stimulation with COS-7 cells co-transfected with the pcDNA3.1 expression vector containing NY-ESO-1 cDNA and pcDNA1Amp containing HLA- B51 cDNA. No specific TNF-alpha release was detected after stimulation with COS-7 cells transfected with HLA-A3 and NY-ESO-1 or with the T cell lines alone.

## Specific cytotoxicity of T cell clone NW1539-CTL-1/1 against NY-ESO-1 p94-102 and naturally processed NY-ESO-1

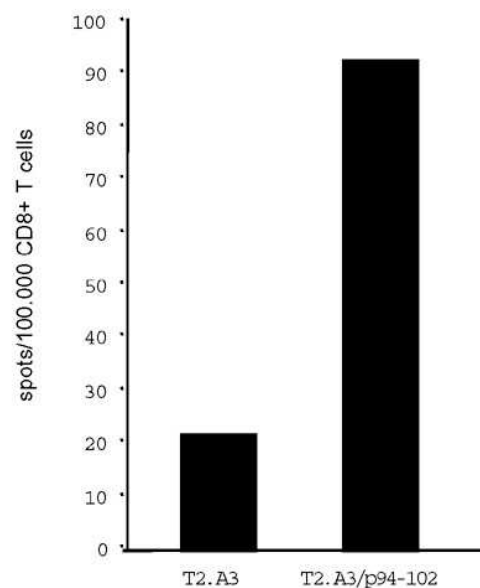
T cell clone NW1539-CTL-1/1 was generated by limiting dilution and repeated *in vitro* stimulation with NY-ESO-1 p94-102 peptide. Cytotoxicity of T cell clone NW1539-CTL-1/1 against NY-ESO-1 p94-102 peptide-pulsed T2.A3 cells, autologous vvESO-transduced NW1539-EBV B cells, the NW1539-MEL-1 melanoma cell line and the allogeneic LB39 melanoma cell line, which shares only the HLA-B51 allele with the NW1539-CTL-1/1 effector cells, is demonstrated in Figure 8. No lysis was observed against untreated T2.A3, K562, and NW1539-EBV B cells.

## Spontaneous *ex vivo* reactivity of CD8<sup>+</sup> NW1539 T cells against NY-ESO-1 p94-102

Unsensitized CD8<sup>+</sup> selected T cells from melanoma patient NW1539 were analyzed for specific recognition of NY-ESO-1 p94-102 in ELISPOT assays. The high number of peptide-specific spots shown in Figure 9 suggests a precursor frequency of NY-ESO-1 p94-102-reactive T cells of approximately 0.7% of the CD8<sup>+</sup> T cell population in this patient. Rare cases of comparable *ex vivo* precursor frequencies against Melan A/MART-1 p26-34 (15, 16) and NY-ESO-1 p157-167 (17) were reported for individual patients during prolonged vaccination with the respective peptides. In this light, the spontaneous CD8<sup>+</sup> T cell reactivity against NY-ESO-1 p94-102 supports previous findings pointing to the strong immunogenicity of NY-ESO-1 in cancer patients.



**Figure 8. Specific cytotoxicity of the T cell clone NW1538-CTL-1/1 against NY-ESO-1 p94-102 and naturally processed NY-ESO-1.** Cytotoxicity of the T cell clone NW1539-CTL-1/1 against NY-ESO-1 p94-102 peptide-pulsed T2.A3 cells, autologous vvESO-transduced NW1539-EBV B cells, NW1539-MEL-1 and LB39 melanoma cells as assessed in a standard  $^{51}$ chromium release assay. No reactivity was found against T2.A3, untreated NW1539-EBV-B cells and K562 cells. Effector cells were used at effector to target ratios of 60:1 (black), 30:1 (checkered), 10:1 (white) and 1:1 (striped).



**Figure 9. Spontaneous *ex vivo* reactivity of CD8+ NW1539 T cells against NY-ESO-1 p94-102 peptide.** ELISPOT analysis of the recognition of T2.A3 target cells unpulsed or pulsed with NY-ESO-1 p94-102 peptide by unsensitized CD8+ selected T cells from patient NW1539.



## Discussion

The cancer/testis antigen NY-ESO-1 is one of the most immunogenic tumor antigens known to date. NY-ESO-1 serum antibodies are detected in half of the patients with advanced NY-ESO-1 expressing malignancies (9, 11). NY-ESO-1-specific CD8<sup>+</sup> T cell responses assessed in a population of HLA-A2<sup>+</sup> patients with advanced NY-ESO-1-expressing cancers showed a close correlation with detectable NY-ESO-1 serum antibody (11, 12). To extend the monitoring of T cell immunity against NY-ESO-1 to epitopes presented by non-HLA-A2 alleles, Gnjjatic *et al.* have introduced a new method that circumvents the need for established tumor and T cell lines, as well as for predefined peptide epitopes presented by respective HLA restriction elements. Antigen-presenting cells transfected with NY-ESO-1 recombinant adenoviral constructs were used to stimulate CD8<sup>+</sup> effector T cells. These effectors were tested for the recognition of autologous or HLA-matched allogeneic NY-ESO-1 vaccinia virus transduced EBV-B cells. Two naturally processed NY-ESO-1 peptides were identified with this approach that are presented by HLA-Cw\*03 (NY-ESO-1 p92-100) and HLA-Cw\*06 (NY-ESO-1 p80-88) (12).

To expand the list of MHC class I-restricted NY-ESO-1 peptides that can be used to monitor NY-ESO-1-specific T cell responses and to vaccinate cancer patients, we selected NY-ESO-1 seropositive patients sharing the HLA-A3 and B51 allele for the analysis of spontaneous NY-ESO-1-specific CD8<sup>+</sup> T cell responses. Ad2/ESO- or NY-ESO-1 p94-102 peptide-sensitized CD8<sup>+</sup> selected effector cells of four patients (NW1539, NW1354, NW923, NW1352) with NY-ESO-1+ cancers specifically recognized HLA-A3<sup>+</sup> and B51<sup>+</sup> T2.A3 cells pulsed with long overlapping 18-mer NY-ESO-1 peptides (NY-ESO-1 p85-102 and p91-108). The long NY-ESO-1 peptides recognized were analyzed for potential binding motifs to HLA-A3 and/or B51. Six NY-ESO-1 9- and 10-mer peptides (Table 1) were tested and peptide p94-102 was recognized by Ad2/ESO-sensitized NW1539 T cells. Analysis of four additional HLA-A3 and/or B51<sup>+</sup> patients confirmed the recognition of NY-ESO-1 p94-102 peptide.

**Table 1. NY-ESO-1 peptides.**

Position	Sequence
85-93	SRLLIFYLA
91-99	YLAMPFATP
92-100	LAMPFATPM
93-101	AMPFATPME
94-102	MPFATPMEA
97-105	ATPMEAEELAR

The NY-ESO-1-specific CD8+ T cell clone NW1539-CTL-1/15 was found to recognize NY-ESO-1 p94-102 peptide-pulsed onto T2 and T2.A3 cells. HLA-B51 is the HLA class I allele shared by both target and effector cells, suggesting that NY-ESO-1 p94-102 is presented by and recognized in the context of HLA-B51. HLA-B51 restriction was confirmed by testing peptide-pulsed target cells expressing different HLA class I alleles for their ability to recognize peptide-presentation CD8+ T cells from HLA-A3-, B51+ melanoma patient NW1274.

Antigenic peptides derived from tumor antigens may be considered for the vaccination of cancer patients if they represent naturally processed epitopes that can be recognized by CD8+ T cells when presented on antigen expressing tumor cells ([18](#), [19](#), [20](#), [21](#), [22](#), [23](#), [24](#), [25](#)). Circumventing the difficulty of establishing tumor cell lines with different tumor antigen- and HLA profiles, the gene of interest can be expressed in monocyte-derived antigen-presenting cells or EBV-B cells and tested for the recognition of naturally processed epitopes by HLA-matched CD8+ effector T cells. Co-transfection experiments with NY-ESO-1 along with HLA-A3 or B51 showed that natural processing of NY-ESO-1 p94-102 was restricted to HLA-B51. NY-ESO-1 p94-102 peptide-presentation CD8+ T cells recognized NY-ESO-1-transduced, autologous, monocyte-derived APCs and the HLA-B51+, NY-ESO-1+ LB39 melanoma cell line, suggesting that NY-ESO-1 p94-102 represents a naturally processed epitope. Recognition of NY-ESO-1 p94-102 peptide by effector T cells derived from HLA-B51 negative individuals may result from cross-presentation of the antigen in the context of an additional HLA class I molecules yet to be defined.

We have recently evaluated the immunological and clinical effects of intradermal immunization with the HLA-A2-restricted NY-ESO-1 epitopes p157-167 (SLLMWITQCFL), p157-165 (SLLMWITQC), and p155-163 (QLSLLMWIT) in a clinical trial ([22](#)). Strong inflammatory delayed-type hypersensitivity reactions associated with primary CD8+ T cell responses against NY-ESO-1 p157-167 and p157-165 were induced in the majority of patients without spontaneous immunity to NY-ESO-1. Stabilization and regression of metastatic disease observed in individual patients coincided with the induction of peptide-specific CD8+ T cell responses *in vivo* ([22](#)). The potential role of the new NY-ESO-1 p94-102 epitope in clinical vaccine trials will be assessed using recombinant HLA-B51/peptide multimers, ELISPOT and cytotoxicity assays to determine the immunogenicity of this peptide in a larger series of NY-ESO-1 seropositive and seronegative, HLA-B51+ cancer patients. If the correlation of spontaneous NY-ESO-1 serum antibody with detectable CD8+ T cell reactivity can be confirmed in HLA-B51+ cancer patients with the new NY-ESO-1 p94-102 epitope, the monitoring of spontaneous and vaccine-induced immune responses against NY-ESO-1 could be extended to HLA-B51+ patients.

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## Materials and methods

### NY-ESO-1 serum antibody

Serum antibody responses against the recombinant NY-ESO-1 protein were tested by standard Western blot analysis and ELISA, using NY-ESO-1 recombinant protein purified from *E. coli* as described (9).

### Patients

Five patients with advanced NY-ESO-1-expressing cancer and detectable NY-ESO-1 serum antibody were selected for the assessment of peptide-specific CD8+ T cell responses. Diagnoses and HLA class I types were as follows: Melanoma NW1539 (A\*03, A\*11; B\*44, B\*51; Cw\*04, Cw\*05); melanoma NW1352 (A\*03, A\*31; B\*15, B\*35; Cw\*03, Cw\*04); urothelial carcinoma NW923 (A\*01, A\*03; B\*51, B\*52; Cw\*06, Cw\*012); non-small cell lung cancer NW1354 (A\*03, A\*11; B\*35, B\*44; Cw\*04, Cw\*016); melanoma NW1274 (A\*2; B\*44, B\*51; Cw\*1-7 not determined). PHA blasts used as antigen-presenting target cells were prepared from peripheral blood lymphocytes (PBL) of two healthy donors, NW1725 (A\*32; B\*51; Cw\*1-7 not determined) and NW1726 (A\*2; B\*7, Bw\*6; Cw\*7).

### Cell lines

The tumor cell lines NW-MEL-38 (A\*01, A\*02; B and C locus not determined), NW-MEL-1539 (A\*03, A\*11; B\*44, B\*51; Cw\*04, Cw\*05), and LB39 (A\*02, A\*24; B\*07, B\*51; Cw\*01, Cw\*07), a generous gift from Dr. Thierry Boon, were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10 mM HEPES buffer, L-arginine (84 mg/l), L-glutamine (584 mg/l), penicillin (10 IU/ml), streptomycin (100 µg/ml), and 10% FCS. LB39 was treated with 100 U/ml IFN-gamma for 5 days to upregulate MHC class I before being used in the cytotoxicity assay. EBV-transformed B lymphocytes, NW115-EBV and MZ1257-EBV, were used as feeder cells for the T cell culture. The mutant cell lines CEMx721.174.T2 (T2) and CEMx721.174.T2 transfected with HLA-A3 (T2.A3), kindly provided by Dr. Vincenzo Cerundolo, were maintained in RPMI 1640 medium supplemented with 10 mM HEPES buffer, L-arginine (242 mg/l), L-asparagine (50 mg/l), L-glutamine (300 mg/l), penicillin (10 IU/ml), streptomycin (100 µg/ml), 1% non-essential amino acids and 10% FCS.

The CD8+ T cell line NW1539-IVS-1 and the T cell clone NW1539-CTL-1/15 were generated using PBLs from patient NW1539 and were used for *in vitro* stimulation of the autologous tumor cell line NW-MEL-1539. The CD8+ T cell clone NW1539-CTL-1/1 was obtained by limiting dilution and repeated *in vitro* stimulations with NY-ESO-1 p94-102.

## Peptides

Peptides 18 amino acids in length with overlapping sequences spanning the entire NY-ESO-1 sequence have been described previously (26). NY-ESO-1 nonamer and decamer peptides were synthesized by Multiple Peptide Systems (San Diego). The purity of the peptides was >95%. The sequences of these peptides are given in Table 1.

## Viral vectors

Adenoviral constructs Ad2/EGFP (encoding green fluorescent protein) and Ad2/ESO (encoding NY-ESO-1) were provided by Genzyme Corporation, Farmington MA. Vaccinia constructs vvWT (wild type vaccinia virus) and vvESO (encoding the full length NY-ESO-1 cDNA) were provided by Therion Biologics, Cambridge MA. These have been described previously (12).

## Presensitization with peptides and adenoviral constructs

Purified CD8+ T lymphocytes were presensitized with peptide-pulsed or adenovirus-infected irradiated autologous PBLs (depleted of CD4+ and CD8+ T cells) as described. Presensitized CD8+ T cells were used as effectors on day 6 for ELISPOT analysis or restimulated on day 7 to assess cytotoxicity against peptide-pulsed T2.A3 cells, adenovirus-infected autologous APCs or melanoma cells in <sup>51</sup>chromium release assays 7 days later.

## Target cells

To generate monocyte-derived, autologous antigen-presenting cells, peripheral blood mononuclear cells (PBMCs) depleted of CD4+ and CD8+ T lymphocytes using magnetic beads (MiniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany) were seeded in 24-well plates at  $4 \times 10^6$  cells/well and allowed to adhere to plastic for 24 h. Non-adherent cells were removed and the remaining cells were used as antigen-presenting cells (APCs). These were cultured with 1000 U/ml GM-CSF (Leukomax, Sandoz, Nürnberg, Germany) and 1000 U/ml IL-4 (Pharma Biotechnologie Hannover, Germany) for 5 days in 2 ml/well X-vivo 15 medium (Bio Whittaker, Walkersville, Maryland, USA). To assess the HLA-B51-restricted presentation of NY-ESO-1, APCs were treated on day 6 of *in vitro* culture with 1000 U/ml IL-4, 1000 U/ml IL-6, 10 ng/ml IL-1 $\beta$ , 10 ng/ml TNF- $\alpha$  (all from Pharma Biotechnologie Hannover, Germany), 1000 U/ml GM-CSF, and 1  $\mu$ g/ml prostaglandin (Sigma Chemical Co., St. Louis, MO). APCs were infected with adenoviral constructs at 1000 infectious units/cell or pulsed with peptides at 10  $\mu$ g/ml and cultured for 24 h. The APCs were then washed twice and used as targets in ELISPOT assays at  $3 \times 10^4$  cells/well.

PHA blasts generated from PBLs by incubation with 0.5  $\mu$ g/ml PHA (Welcome) were pulsed with 10  $\mu$ g/ml peptide. EBV-B cells were either pulsed with 10  $\mu$ g/ml peptide or infected overnight with adeno- or vaccinia wild type or recombinant for NY-ESO-1 at 30 pfu/cell in 300  $\mu$ l serum-free medium.

## ELISPOT assays

Effector cells, either CD8+ T lymphocytes presensitized as described above ( $2.5 \times 10^4$  cells/well) or T cell clones (1000 cells/well), were added to 96-well flat-bottom nitrocellulose plates (MAHA S45 10, Millipore, Bedford MA, USA) coated with 5  $\mu$ g/ml anti-IFN- $\gamma$  antibody (Hölzel Diagnostic, Köln, Germany) in a final volume of 100

$\mu$ l. Irradiated T2 and T2.A3 cells were pulsed with NY-ESO-1 peptides at a concentration of 10  $\mu$ g/ml, washed and added to the effector cells at  $5 \times 10^4$  cells/well. Plates were incubated for 16 h at 37°C, washed extensively (6 times with 0.05% Tween 20 in PBS) and 0.5  $\mu$ g/ml biotinylated anti-IFN-gamma detection antibody (Hözel Diagnostic, Köln, Germany) was added. Following incubation for 2 h at 37°C, the plates were washed and developed with ABC-AP Vectastain (Vector, Burlingame, CA, USA) for 1 h. Following the addition of substrate (BCIP/NPT) and a 10 min incubation, the plates were prepared for microscopy and the number of blue spots/well, corresponding to the proportion of NY-ESO-1-specific CD8+ T cells/well, were counted.

### Cytotoxicity assays

Cytotoxicity against peptide-pulsed T2.A3 cells, NY-ESO-1-transduced EBV-B cells, and tumor cell lines was determined in standard chromium release assays as described (11).

### COS cell transfections

The full-length NY-ESO-1 sequence was isolated from the corresponding pQE9 clone and cloned into the *Bam*HI-*Hind*III sites of the pcDNA3.1(-) vector (Invitrogen). Transfection of COS-7 cells was carried out as described (27). Briefly,  $2 \times 10^4$  COS-7 cells were transfected with 150 ng pcDNA3.1(-) containing NY-ESO-1 cDNA and 150 ng pcDNA1Amp containing HLA-A3 or HLA-B51 cDNA using the DEAE-dextran-chloroquine method. The HLA-A3 and HLA-B51 cDNAs were kindly provided by Drs. Thomas Wölfel and Thierry Boon respectively. Transfectants were incubated at 37°C for 48 h and tested in a T cell stimulation assay after 24 h.

### Immunoscreening of NY-ESO-1 transfectants

Transfectants were tested for their ability to stimulate TNF-alpha production by the CD8+ T cell lines NW923-IVS-1 and NW1274-IVS-1 (generated by repeated *in vitro* stimulation with NY-ESO-1 p94-102) and by NW1539-IVS-1 (generated by repeated *in vitro* stimulation with the autologous tumor cell line NW-MEL-1539) as described (27). Briefly, 5000 CD8+ T cells in 100  $\mu$ l RPMI supplemented with 10% human serum and 25 U/ml rhIL-2 were added to microwells containing COS-7 transfectants. After 24 h, 50  $\mu$ l of supernatant were collected and the TNF-alpha content determined by testing the cytotoxicity against WEHI 164 clone 13 cells in an MTT colorimetric assay.

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